Development of Inactivated and Recombinant Vaccine for Vibriosis in Asian seabass (*Lates calcarifer*, Bloch, 1790) and its Comparative Evaluation with Reference to Immune Related Genes.

SYNOPSIS OF THE THESIS

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Chapter 1. General introduction and review of literature

1.1 General introduction

The nutritional requirements of the global population can and will predominantly be fulfilled by aquaculture-derived fish and other aquatic products, while simultaneously catering to the food security needs of the most impoverished segments of the population. Aquaculture and fisheries have become an important source of income generation, food and nutrition security for millions of people across the world. The global fish production in 2020 was 178 million tonnes, and the contribution of aquaculture was 87.5 million tonnes. About 66 per cent of world aquaculture production of aquatic organisms was contributed by finfish culture (FAO, 2022).

Asian seabass (*Lates calcarifer* (Bloch, 1790)), commonly called barramundi or giant sea perch, is a teleost fish belonging to the order Carangiformes (Fricke et al., 2023). This species is euryhaline, catadromous and cannibalistic (Moore, 1979). They are bottom-living, medium to large-sized fishes (350g to 3 kg) present in lagoons, estuaries and coastal seas at depths up to 50 metres (Mathew et al., 2009). It has wide geographical distribution, including the whole of Southeast Asian countries, western parts of India, Papua New Guinea and Northern Australia (Yue et al., 2012).

Disease, is a major hurdle in aquaculture, causing significant economic losses. Despite the widespread hatchery production and commercial expansion of Asian seabass, emerging and re-emerging diseases pose a significant threat to the industry. Its sustained production is affected by bacterial, viral, fungal and parasitic diseases. Several diseases pose significant threats to Asian seabass aquaculture, with vibriosis being a major concern. This bacterial infection can lead to high mortality rates, especially in grow-out systems where younger fish are more susceptible. Moreover, in vibriosis-endemic waters, the production cost of marine cage-cultured Asian seabass can increase significantly due to mortality, treatment, and diagnosis expenses (Ina-Salwany et al., 2019).

Vibriosis, caused by bacteria from the Vibrionaceae family, is a significant systemic bacterial disease affecting both wild and farmed marine fish and shellfish globally (Dong et al., 2017). It is one of the most prevalent and destructive bacterial infections in aquaculture, leading to substantial economic losses (Mancuso et al., 2015). The disease is triggered by environmental factors such as fluctuations in water quality and overcrowding (Kumar et al., 2015). *Vibrio* species, including *V. alginolyticus, V. salmonicida*, *V. anguillarum*, and others, are responsible for outbreaks in various fish life stages, with their occurrence influenced by

seasonal and temperature variations (Amin et al., 2017). The disease is widespread, especially in tropical and temperate regions like Asia, North America, and Europe (Ina-Salwany et al., 2019).

Vibrio alginolyticus is a gram-negative, halophilic, facultative anaerobic, and motile bacterium belonging to the family Vibrionaceae, with a global distribution (Guo et al., 2018). It is commonly found in marine and brackish water environments (Lajnef et al., 2012). Although initially considered part of the standard marine flora (Carli et al., 1993), it is now recognized as a potential pathogen. Currently, this bacterium is a significant concern in aquaculture due to its pathogenicity in various marine species, particularly fish and shellfish. *V. alginolyticus* causes vibriosis, a disease that leads to high mortality rates in infected aquatic organisms, posing a serious threat to the aquaculture industry. The infection manifests through symptoms such as skin lesions, fin rot, and hemorrhaging, severely impacting the health and commercial value of affected species like *Oncorhynchus mykiss* (Austin et al., 1993), *Epinephelus coioides* (Lee, 1995), *Sparus aurata* (Balebona et al., 1998), *Dicentrachus labrax* (Kahla et al., 2006)), *Lates calcarifer* (Krupesha et al., 2012), and *Rachycentron canadum* (Rajan et al., 2001; Rameshkumar et al., 2014), leading to significant economic losses.

Antibiotics are widely used in aquaculture to control bacterial infections like vibriosis, but their overuse has led to the emergence of antibiotic-resistant bacteria, posing a serious public health threat by rendering treatments ineffective (Pepi & Focardi, 2021; Serwecińska, 2020). To mitigate this issue, vaccination offers a safer and more sustainable alternative, providing long-term immunity without contributing to antibiotic resistance (Rosini et al., 2020; Xu et al., 2022). Vaccination has proven effective in controlling diseases like vibriosis, reducing the reliance on antibiotics in aquaculture (Ma et al., 2019). The use of transcriptomics after a vibriosis infection in fish allows to understand the host's immune response at the molecular level. This helps identify key genes involved in immune pathways, aiding the development of vaccines that stimulate specific protective responses. Whole genome sequencing of pathogenic bacteria like *Vibrio* species provides insights into virulence factors, antibiotic resistance genes, and antigenic proteins. This comprehensive genetic understanding helps in designing targeted vaccines by identifying bacterial components that can trigger a strong immune response without causing disease. By promoting vaccination and implementing strong biosecurity measures, aquaculture can shift towards more sustainable and health-conscious disease management strategies.

1.2 objectives of the study

- Performing whole genome sequencing of *V. alginolyticus* to elucidate the presence of virulent and antimicrobial-resistant genes.
- Histopathology and Transcriptome profiling of Asian seabass immune organs in both uninfected and *V. alginolyticus* infected states.
- Comparative evaluation of the different vaccines and route of vaccination (Bath, dip, oral, intra peritoneal, intramuscular).
- To develop inactivated and recombinant vaccines against *V. alginolyticus* for Asian sea bass.
- Real time expression profiling of immune related genes in vaccinated and non-vaccinated fishes.

1.2 Review of literature

1.3.1. Vaccination approaches against Vibriosis in aquaculture

1.3.1.1. Inactivated Vaccines

Inactivated vaccines utilize infectious bacteria or viruses that have been inactivated through physical or chemical means, maintaining their antigenicity while losing pathogenicity (Tlaxca et al., 2015). In aquaculture, studies on inactivated vaccines have shown promising results. For example, intraperitoneal injection of formalin-inactivated *Vibrio harveyi* in Asian seabass induced strong antibody responses and enhanced bacteriostatic activity, although a challenge test was not included (Crosbie & Nowak, 2004). Further research using a formalin-inactivated *Vibrio anguillarum* vaccine in Asian seabass demonstrated survival rates of 60%, 75%, and 62.5% across different fish size categories (Kumaran et al., 2010). Similarly, tilapia vaccinated with a formalin-inactivated whole-cell *Vibrio vulnificus* vaccine showed a relative percent survival (RPS) of 88% (Shoemaker et al., 2011). Recent studies have confirmed that formalin-killed vaccines offer effective protection against vibriosis in Asian seabass, with oral booster regimens yielding survival rates as high as 84.62% (Ahangarzadeh et al., 2022, 2023). Oral vaccines have also shown potential, as demonstrated by increased survival rates of 70%, 75%, and 85% in Asian seabass challenged with *V. alginolyticus*, V. *harveyi*, and *V. parahaemolyticus* (Mohamad et al., 2022). Field studies also support the efficacy of feed-based inactivated vaccines, with enhanced immune responses and survival rates of 71.3% in cage-cultured Asian seabass (Amir-Danial et al., 2022). Further innovations, such as immersion prime-oral booster vaccination and the use of oxygen nanobubbles, continue to show promise for enhanced vaccine delivery and immune responses in aquaculture (Lan et al., 2024; Thu Lan et al., 2024). These studies underscore the effectiveness of inactivated vaccines, particularly in preventing vibriosis, while advancing sustainable disease control strategies in aquaculture.

1.3.1.2. Subunit vaccines

Subunit vaccines are created by introducing genes encoding specific antigens from pathogens into expression systems, purifying these antigens, and formulating them into vaccines that stimulate immune responses (Xing et al., 2017). These vaccines have shown promise in aquaculture for preventing vibriosis. For instance, recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been effective in turbot and tilapia against *Edwardsiella tarda*

and *E. ictaluri*, achieving protection rates of over 71.4% (Liang et al., 2011; Trung et al., 2014). A study on the OmpK gene from *Vibrio anguillarum* showed a relative percentage survival (RPS) of 67.8% in vaccinated carp (Hamod et al., 2012), while the OmpU gene from *V. alginolyticus* provided 93.33% protection in crimson snapper (Cai et al., 2013). Further, LamB gene-based vaccines protected zebrafish against multiple *Vibrio* species (Lun et al., 2014), and *Streptococcus iniae* GAPDH demonstrated cross-protection in zebrafish (Kim et al., 2015). Multivalent vaccines combining different antigens, such as FlaC, OmpK, and OmpR proteins from *V. anguillarum*, increased RPS to 78.95% in flounders, offering better protection compared to formalin-inactivated vaccines (Xing et al., 2018). Similarly, recombinant OmpK protein provided 100% protection against *V. alginolyticus* in hybrid grouper (Nehlah et al., 2017). In Asian seabass, recombinant proteins r-OmpK and r-DnaJ offered RPS values of 90% and 100% against *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*, making them promising candidates for vibriosis control in marine fish (Silvaraj et al., 2020).

1.3.2. Transcriptomic analysis of immune responses in Asian seabass and other fish following *V. alginolyticus* and related infections

The transcriptome analysis of response of Asian seabass to *V. alginolyticus* infection is presented for the first time in this investigation. While transcriptomic analyses concerning *V. alginolyticus* infections in fishes are limited. Wang et al. (2014) conducted a transcriptome analysis to elucidate the effects of *V. alginolyticus* infection on the innate immune response in grouper larvae (*Epinephelus coioides*), focusing on the complement pathway. Employing high-throughput sequencing, they identified 3,977 differentially expressed genes (DEGs), revealing significant changes in genes related to the complement and coagulation cascades, indicating a robust immune response. The research demonstrated a time-dependent upregulation of specific genes within the complement pathway, underscoring its crucial role in larval defense mechanisms against infection. In a subsequent study, Wang et al. (2016) undertook a transcriptome analysis to investigate the immune response of giant grouper larvae (*Epinephelus lanceolatus*) to *V. alginolyticus* infection, focusing on TLR5-mediated cytokine induction. This study identified 5,913 unigenes, revealing significant alterations in the expression levels of immune-related genes, particularly those associated with cytokines, chemokines, and the complement system.

The number of investigations focused on transcriptome analysis in Asian seabass remains limited. Liu et al. (2016) conducted a transcriptomic study to explore the molecular response of Asian seabass epithelial cells to nervous necrosis virus (NNV) infection. The majority of DEGs, including key innate immunity-related genes, exhibited upregulation, suggesting a robust immune response. Angsujinda et al. (2020) focused on understanding the transcriptomic response of Asian seabass infected with red-spotted grouper nervous necrosis virus (RGNNV). Transcriptome analysis was performed on the head kidneys of both RGNNV-infected and mock-infected fish. The DEGs were annotated, categorized, and subsequently validated using quantitative PCR (qPCR).

1.3.3. Studies on immune related gene expression

Numerous investigations are undertaken to clarify the function of distinct genes in the context of pathogen-host interactions as well as the administration of various vaccines. The investigation into the live attenuated vaccine of *V. alginolyticus* mutant in orange-spotted grouper highlights the upregulation of immune-related genes following vaccination. Specifically, MHC-Iα and MHC-IIα, crucial for humoral and cellular-mediated immunity, respectively, along with IgM, the primary antibody in the early immune response, and the pro-inflammatory cytokine IL-1β, were significantly elevated in the spleen and head kidney of vaccinated fish compared to controls (Pang et al., 2018). In another study on the efficacy of vaccination against vibriosis in Asian seabass against *V. harveyi* highlights several immune-related genes and their expression patterns in response to vaccination and infection. Interleukin-10 (IL-10), Interleukin-8 (IL-8), Interleukin-1 beta (IL-1ß), MyD88, Toll-like receptor 2 (TLR2) and Major Histocompatibility Complex I (MHCI) were studied for expression analysis (Silvaraj et al., 2020).

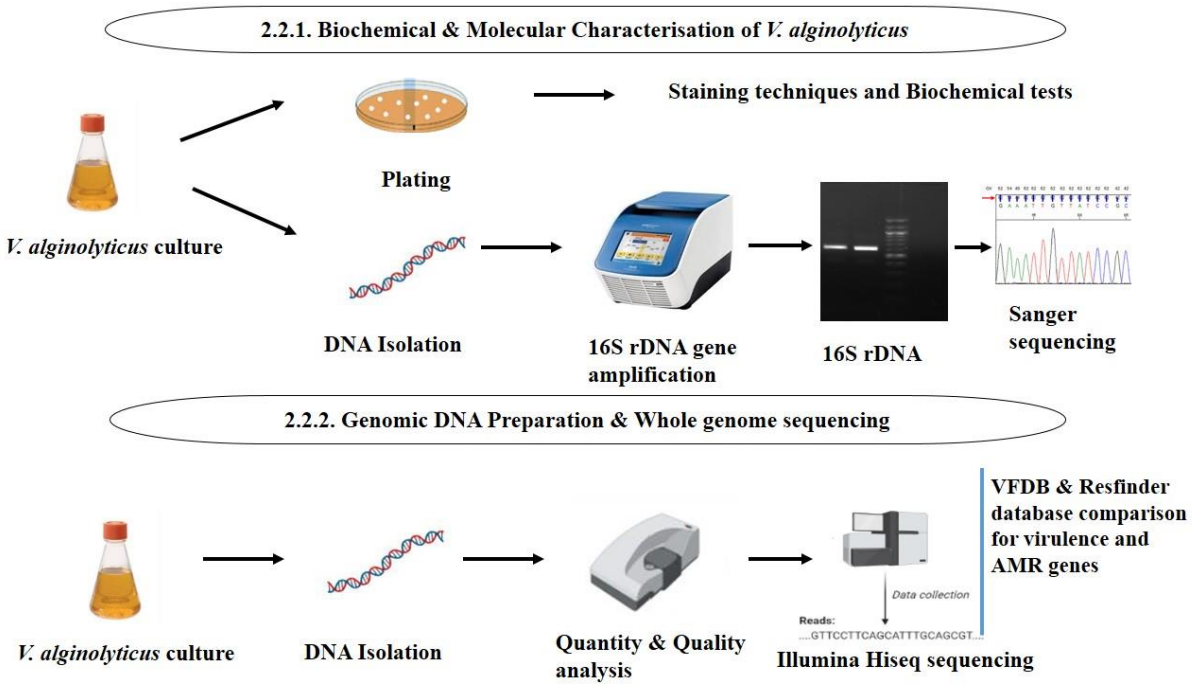
Chapter 2. Complete genome sequencing and analysis of *Vibrio alginolyticus* (CMFRI-JF260912)

2.1. Introduction

As a potential candidate for aquaculture vaccine development, it is crucial to thoroughly understand the characteristics of *V. alginolyticus*, both microbiologically and molecularly. Whole genome sequencing (WGS) can shed light on the genetic makeup of *V. alginolyticus* and enhance our understanding of its pathogenic potential in aquaculture by revealing virulence factors and antimicrobial resistance genes. Compared to traditional Sanger sequencing, next-generation sequencing technologies significantly reduce the cost and time required for genome sequencing. The whole genomes of many marine pathogenic *Vibrio* bacteria have been sequenced and used for various applications (Carmona et al., 2021; Kalatzis et al., 2019; Liu et al., 2017; Xu et al., 2018).

Horizontal gene transfer (HGT) plays a critical role in the acquisition of virulence factors and resistance genes, serving as a major driving force for the emergence and evolution of antibiotic-resistant strains (Wang et al., 2016). Therefore, it is crucial to comprehend the genomic features and virulence factors of *V. alginolyticus* to gain insights into its pathogenicity and antibiotic resistance mechanisms. Identifying antimicrobial resistance genes is particularly important, as it informs the development of effective vaccines and treatment strategies in aquaculture (Kamile & Tenover, 2011; Aoki et al., 2011; Lou et al., 2016).

2.2. Materials and method



2.3. Results

2.3.1. Genome Characteristics:

- The genome of *Vibrio alginolyticus* (CMFRI-JF260912) consists of two circular chromosomes:
    - **Chromosome I:** 3,287,226 bp, 54.5% GC content, 3,790 predicted ORFs.
    - **Chromosome II:** 1,764,503 bp, 50.69% GC content, 2,065 predicted ORFs.
- A total of **4,510 genes** were predicted from the genome assembly.

2.3.2. Comparative Genome Analysis:

- The isolate is phylogenetically similar to *Vibrio alginolyticus* (ATCC 33787).
- A phylogenetic tree was created using single-copy orthologous genes from 10 *Vibrio* species and an out group (*Photobacterium damselae* subsp. *damselae*), indicating a close evolutionary relationship with *Vibrio natriegens*.

2.3.3. Gene Ontology (GO) and KEGG Analysis:

- **GO Database:** 39.02% (1,760 genes) annotated, with functions linked to transcription (9.4%), membrane components (42.43%), and ATP binding (8.1%).
- **KEGG Database:** 59.44% (2,681 genes) annotated, with high representation in carbohydrate metabolism (10.66%), amino acid metabolism (9.4%), metabolism of cofactors and vitamins (6.7%), and membrane transport (6.67%).

2.3.4. Virulence and Antimicrobial Resistance Genes:

- **Virulence Factors:** 80% of known virulence elements of *V. alginolyticus* were detected, including Type II (T2SS) and Type III secretion systems (T3SS), LuxS gene, and adeG gene on Chromosome I, and tlh gene on Chromosome II.
- **Antimicrobial Resistance Genes:** Four notable genes homologous to antimicrobial resistance genes were identified:
    - **Tet 35:** Tetracycline efflux transporter (96.06% identity).
    - **Chloramphenicol O-acetyltransferase:** (99.09% identity).
    - **CARB-42:** Class A beta-lactamase (100% identity).
    - **Tet 34:** Oxytetracycline resistance (92.21% identity).

2.4. Discussion

The genomic analysis of *V. alginolyticus* revealed key differences between its two chromosomes when compared to related species such as *Vibrio cholerae* and *Vibrio parahaemolyticus* (Tagomori et al., 2002; Makino et al., 2003). This suggests distinct functional roles for the two chromosomes, with the conserved architecture of Chromosome I linked to essential functions like growth and metabolism, while Chromosome II's variability is likely associated with environmental adaptation (Makino et al., 2003; Thompson et al., 2009).

A comparative genome analysis of the current isolate revealed a close phylogenetic relationship with *Vibrio natriegens*. The analysis identified numerous virulence factors, crucial for pathogenicity, responsible for all five stages of infection: adhesion, invasion, colonization, proliferation, and toxin production. Approximately 80% of previously reported virulence factors of *V. alginolyticus* were detected in this strain. Wang et al. (2016) similarly identified multiple virulence factors, including microbial collagenase, leukocidin, hemolysin, RTX toxin-related proteins, and zonular occludens toxin in *V. alginolyticus* isolated from seawater. Furthermore, two Type VI Secretion System (T6SS) gene clusters were found in one of the chromosomes of their isolates. The current study also detected Type II (T2SS) and Type III Secretion Systems (T3SS) on Chromosome I. T2SS facilitates the secretion of hydrolytic enzymes and toxins, aiding in tissue degradation and immune evasion (Korotkov & Sandkvist, 2019). T3SS, on the other hand, delivers effector proteins directly into host cells, enabling bacterial colonization and intracellular survival (Coburn et al., 2007). The regulation of these systems is influenced by environmental conditions and transcriptional factors (Ramamurthy et al., 2020), with T2SS aiding in nutrient acquisition and T3SS promoting host cell invasion (Zhao et al., 2018). These systems are crucial in the virulence of *V. alginolyticus*, potentially influencing fish-bacteria interactions across various habitats. The LuxS quorum sensing (QS) system, located on Chromosome I, plays a key regulatory role in virulence factor expression in many pathogenic bacteria. LuxS synthesizes an autoinducer molecule (AI-2) that facilitates intra- and inter-species communication (Jensen et al., 2013). This AI-2 molecule activates genes related to toxin production and biofilm formation, aiding in pathogenicity (Wang et al., 2013). The *adeG* gene, also located on Chromosome I, is involved in biofilm formation, which enhances bacterial adhesion to host surfaces, a critical step in infection (Chen et al., 2008). In addition, the *tlh* gene on Chromosome II encodes a thermolabile hemolysin, a widely distributed virulence component among pathogenic *Vibrio* species (Zhang & Austin, 2005). Hemolysins are associated with apoptotic events in host

cells, as seen in studies involving *V. vulnificus* and *V. parahaemolyticus* (Naim et al., 2001). The study also uncovered antimicrobial resistance (AMR) genes in the isolate, conferring resistance to tetracycline, chloramphenicol, carbenicillin, and oxytetracycline. While *Vibrio* species are generally susceptible to antimicrobials, there have been increasing reports of resistance to multiple drug classes, including beta-lactams and tetracyclines (Zanetti et al., 2001; Dutta et al., 2021).

These findings enhance the understanding of *V. alginolyticus* pathogenesis and antimicrobial resistance, particularly in the context of marine aquaculture systems, where such bacteria may pose a significant threat. The insights into virulence and resistance mechanisms can facilitate the development of targeted interventions to mitigate the risks posed by these pathogens.

Chapter 3. Comparative histopathology and transcriptome analysis of vital organs in healthy Asian seabass and those infected with *Vibrio alginolyticus*.

3.1. Introduction

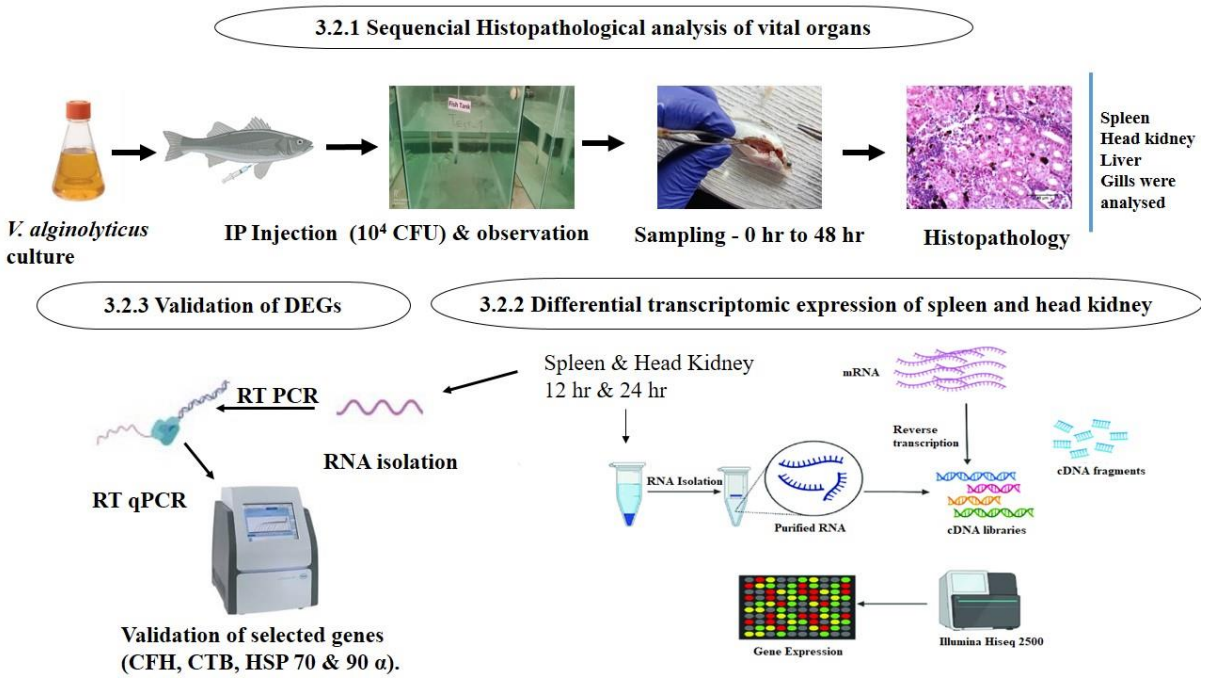
Understanding histopathology and lesion development has significantly contributed to elucidating the pathogenesis of vibriosis in finfish. Researchers report that typical clinical symptoms of affected fish include anorexia, lethargy, skin darkening, tail and fin rot, eye opacity, generalized erythema, inflammation, liquefaction of internal organs, hemorrhagic ulcers, and necrosis across vital organs (Ransangan et al., 2012; Ransangan & Mustafa, 2009; Talpur et al., 2013; Yanuhar et al., 2022). Though there have been a few studies undertaken on the clinical signs and descriptions of internal lesions in naturally and experimentally infected fishes, information on the histopathological changes during the progression of this disease is scarce; such information is valuable for making predictions about the prognosis.

The transcriptome has emerged as a valuable tool to explore critical pathways involved in development, cellular fate, physiology, activity, and disease progression. RNA sequencing (RNA-seq) using next-generation sequencing (NGS) has become the go-to method for transcriptome analysis. Moreover, developments in bioinformatics have strongly supported RNA-Seq technology (Wang et al., 2009). RNA-Seq has gained widespread attention in aquaculture and has been utilized in numerous species and applications, particularly in immunology studies. Researchers have employed RNA-seq to unravel immune mechanisms in various aquaculture species during pathogenic infections. Such transcriptome profiling has revealed the diverse array of immune-related genes in different fish species following bacterial infections (Maekawa et al., 2019a). Therefore, expanding our knowledge of transcriptomic immune responses in diverse fish species against various pathogens is essential.

As outlined in the previous chapter's detailed examination of the pathogen, it is equally important to comprehensively analyze the host and host–pathogen interaction, especially at the tissue-specific level using both microscopic and molecular methods. This study presents a sequential, in-depth characterization of histopathological changes in Asian seabass over 0 to 48 hours following intraperitoneal injection with the pathogen. While the extent of immune responses in Asian seabass against *V. alginolyticus* and their comparability across other aquaculture species remain unclear, we conducted transcriptome profiling of these organs at

two time points selected based on histopathological observations that were most indicative of the host response. The transcriptome sequences and differentially expressed genes generated in this study provide a valuable resource for future research and the development of effective strategies to combat *V. alginolyticus.*

3.2. Materials and methods



3.3. Results

3.3.1. Gross signs and clinical pathology of infected fishes

Clinical signs observed in experimentally infected Asian seabass included lethargy, anorexia, abnormal swimming patterns (such as spinning) within 24 hours post-challenge (HPC). Haemorrhagic lesions on the skin, abdominal distension, exophthalmia, darkened skin, and mortality were seen within 48 HPC.

3.3.2. Histopathological Findings

No significant changes were observed between 0 to 10 HPC. From 12 hours and until 48 hours, the spleen, liver, and kidney showed lesions in the majority of examined fishes.

The spleen of the control fish revealed normal structure of the red pulp with compact ellipsoids and a few melanomacrophage centres (MMC). At 10-48 HPC, splenomegaly with a massive expansion of red pulp by many erythrocytes and ellipsoidal compression of white

pulp were observed. At 12 HPC, approximately 95–100% of the spleen showed an increase in red pulp and accumulation of erythrocytes in the white pulp, increased MMC and hemosiderin deposits. At 24 HPC, loosening of tissue structure and haemorrhage in splenic parenchyma in addition to hemosiderosis were seen. While at 48 HPC, severe splenitis with diffuse fibrinoid necrosis of ellipsoidal sheath and aggregation of inflammatory cells was observed. Hemosiderosis was seen in the majority of examined fish at different time point

Kidneys of the control group exhibited normal organization of nephrons and tubules with a few MMC scattered in the parenchyma. At 12 HPC, approximately 75% of kidney showed haemorrhage, infiltration with inflammatory cells in the interstitium, tubular degeneration, and hemosiderin deposits. At 24 HPC, diffuse necrosis of renal tubule, increase in the number of hemosiderin deposits, loosening of tissue structure and severe haemorrhage were seen. At 48 HPC, severe interstitial nephritis, haemorrhage, increased MMC, hemosiderin deposits, extensive congestion and haemorrhage were seen.

Normal sinusoids and polygonal hepatocytes with mild fatty change were seen in the control group. Only mild lesions were observed in the liver at 2 - 10 HPC. At 12 HPC, the liver showed moderate congestion, and haemorrhage. At 24 HPC, there was pyknosis, severe haemorrhage and congestion. After 48 HPC, liver tissue shows hydrophic degeneration of hepatic cells, cellular swelling, and hepatic congestion with extremely dilated sinusoidal space and congestion in the pancreatic tissue.

Normal primary and secondary lamellae were seen in the gills of control group. At 2–10 HPC, no detectable lesions were observed in any of the examined Seabass. From 12 to 24 HPC, sloughed epithelial cells of secondary lamellae were seen in the majority of examined gills. At 24 and 48 HPC, thickening of both primary and secondary lamellae in 75–80% of the gill lamellar surface, in addition to clubbing of the secondary and primary lamellae, were observed.

3.3.3. Assembly and functional annotation of read transcript sequences

This study performed transcriptome analysis using an Illumina HiSeq™ 2500 platform on head kidney and spleen samples from two groups: one injected with *V. alginolyticus* and a control group injected with PBS. After removing low-quality sequences, over 342,312 clean reads were generated with a high percentage of Q20 and Q30 values in all samples. Using *de novo* assembly, a total of 91,819 unigenes were obtained with an N50 length of 1,515 bp and a GC content of 42.66%.

3.3.4. Differentially expressed genes after *Vibrio alginolyticus* challenge

A total of 5,711 differentially expressed genes (DEGs) were identified across all samples. The relative expression levels of genes in the *V. alginolyticus* group were compared to the PBS-treated groups to determine DEGs in each sample and at each hour post-infection (hpi). The number of total DEGs in the head kidney at 12 hpi, head kidney at 24 hpi, spleen at 12 hpi, and spleen at 24 hpi were 1,703 (938 upregulated and 765 downregulated genes), 1,367 (752 upregulated and 615 downregulated genes), 1,116 (695 upregulated and 471 downregulated genes), and 1,475 (895 upregulated and 580 downregulated genes), respectively. Clustering analysis using a heatmap revealed categorization by tissue rather than by hours post-infection.

3.3.5. Validation of differentially expressed gene by RT-qPCR

We utilized RNA-seq data from the NCBI database to identify mRNA sequences of immune-related genes affected by *V. alginolyticus* infection. Our analysis found that the expression levels of several genes were altered in response to the infection. For real-time validation, we considered a p-value of 0.05. From this, four DEGs found to be upregulated at all four time intervals in both tissues are Complement Factor-H, Cystatin B, Heat Shock Protein 70 (HSP70), and Heat Shock Protein 90 Alpha (HSP90α). These DEGs were associated with immune-related pathways, including cytokine-cytokine receptor interaction, hematopoietic cell lineage, and complement and coagulation cascades. To validate our findings, we performed RT-qPCR analysis on the head kidney and spleen. The expression levels of each gene were consistent with the RNA-seq data, indicating the reliability of the transcriptome information.

Complement Factor H: Twelve hours post-infection, complement factor H expression in the spleen showed a 4.64-fold increase in the transcriptome analysis, which was further validated by real-time PCR, indicating a 2.99-fold upregulation. At the 24-hour mark, the transcriptome analysis revealed a 5.3-fold increase, while real-time PCR validation showed a 2.33-fold upregulation, confirming the sustained activation of this gene in response to infection.

Cystatin B: In response to infection, cystatin B expression was upregulated 4.26-fold in transcriptome analysis and 2.90-fold according to real-time PCR 12 hours post-infection. After 24 hours, the transcriptome analysis showed a 4.55-fold increase, with a 1.45-fold upregulation validated by real-time PCR, highlighting its role in the immune response over time.

Heat Shock Protein 70 (HSP70): Heat shock protein 70 (HSP70) exhibited a 4.2-fold upregulation in the transcriptome analysis and a 2.64-fold increase in real-time PCR

validation 12 hours post-infection. The expression remained elevated at 24 hours, with a 4.17-fold increase in transcriptome analysis and a 2.7-fold upregulation in real-time validation, indicating its involvement in the cellular stress response during infection.

Heat Shock Protein 90 Alpha (HSP90α): HSP90α was upregulated by 4.09-fold in transcriptome analysis and 2.42-fold in real-time PCR validation at the 12-hour post-infection point. After 24 hours, the transcriptome analysis showed a 3.8-fold increase, while real-time PCR indicated a 1.44-fold upregulation, suggesting a sustained but diminishing response to infection over time.

3.4. Discussion

Opportunistic bacterial diseases pose a significant threat to the sustainability of marine cage fish farming. Natural disease outbreaks in aquaculture are frequently associated with concurrent infections involving homologous or heterologous pathogens, which can either amplify or mitigate the severity of the disease caused by the primary pathogen (Kotob et al., 2016). Experimental infection models offer an advantage in studying these diseases by replicating the infections consistently, enabling the sequential tracking of histopathological changes over time (Conforto et al., 2021). This chapter describes the sequential histopathological changes in the spleen, head kidney, liver, and gills and transcriptome profiling of head kidney and spleen Asian seabass after being experimentally challenged with a virulent strain of *V. alginolyticus*. Juvenile fish, which are more susceptible to *Vibrio* infections due to their relatively lower immune resistance (Ina-Salwany et al., 2019; Mohamad et al., 2019; Shen et al., 2017), were selected for this study.

Asian seabass exhibited clinical signs such as reduced activity, loss of appetite, abnormal swimming behavior, and hemorrhagic lesions within 24-48 hours post-challenge. These findings are consistent with other studies on *Vibrio* infections in marine species (Martins et al., 2010; Rameshkumar et al., 2017). Hemorrhagic lesions, a common sign of vibriosis, were also observed internally, similar to previous studies on seabass naturally infected with *V. alginolyticus* (Krupesha et al., 2012). Histopathological analysis revealed early circulatory changes like blood congestion, hemorrhage, and hemosiderin deposits in all studied organs. Inflammatory reactions were observed only in the kidney and spleen, with the spleen showing a delayed inflammatory response compared to the kidney. These findings align with previous research on *Vibrio* infections in other fish species (Mahmoud et al., 2018; Azad et al., 2004; Dong et al., 2017). In contrast, no inflammatory lesions were observed in the liver or gills, which may be due to the route of infection or host-specific responses to *Vibrio* species (Manchanayake et al., 2023; Sumithra et al., 2019; Martins et al., 2010). The septicemic

nature of *Vibrio* infections, which leads to the circulation of bacterial toxins throughout the host, underscores the severe pathogenicity of *V. alginolyticus*. Significant damage to internal organs, particularly the spleen, kidneys, and liver, highlights the need for further research on disease prevention strategies, such as vaccination and improved aquaculture practices (Ina-Salwany et al., 2019).

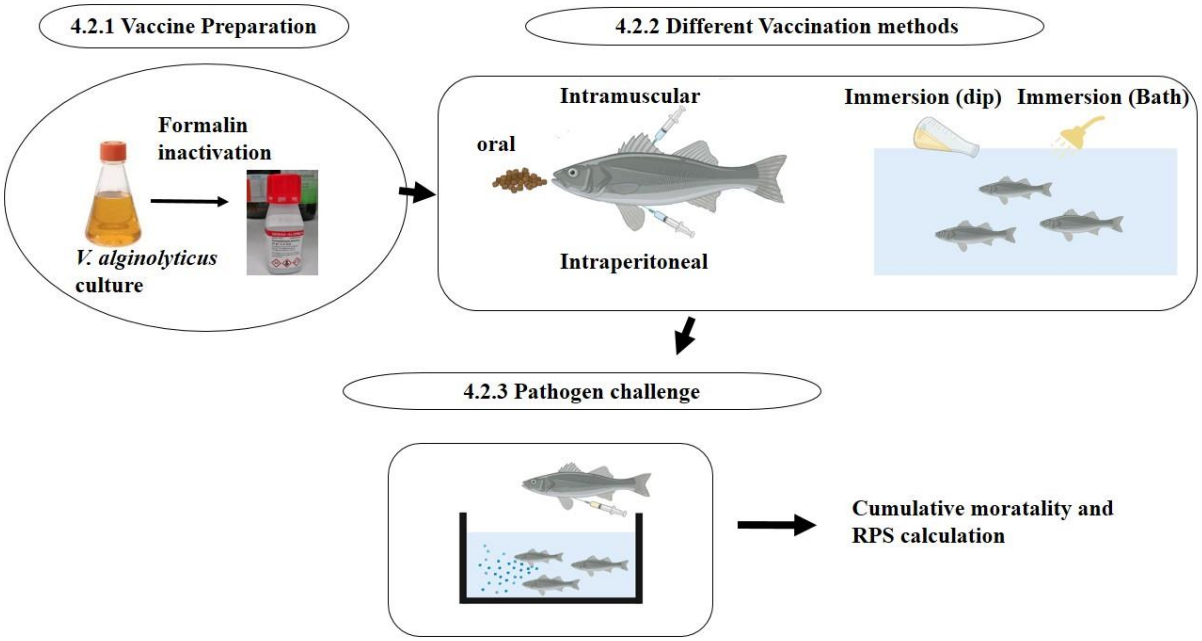
In terms of molecular mechanisms, transcriptome analysis of the head kidney and spleen at 12 and 24 hours post-infection revealed dynamic changes in gene expression. The number of differentially expressed genes (DEGs) was greater in the head kidney at 12 hours but shifted to the spleen at 24 hours. This organ-specific response suggests that different tissues may play pivotal roles at distinct infection stages (Wang et al., 2022; Maekawa et al., 2019b; Peng et al., 2019). Complement factor H (CFH) and other immune-related proteins such as cystatin B and heat shock proteins (HSPs) were implicated in the fish's immune response, offering insights into potential molecular targets for enhancing disease resistance (Qi et al., 2018; Zhou et al., 2024; Li & Zhang, 2022; Wickramasinghe et al., 2020; Chen et al., 2014). CFH, a regulatory protein, plays a crucial role in preventing excessive complement activation, a defence mechanism seen in various teleosts. CFH has been shown to bind to bacterial surfaces, potentially aiding pathogens in evading complement-mediated lysis (Parente et al., 2017). Its upregulation during infections suggests its dual role in immune defence and pathogen interaction. Similarly, cystatin B, a protease inhibitor, enhances immune responses in teleosts by inhibiting cysteine proteases, thus protecting against bacterial and viral infections (Kodagoda et al., 2022; Yu et al., 2018). HSPs, especially HSP70 and HSP90α, were also found to play significant roles in stress responses and immune activation. HSP70 is a well-established marker of cellular stress and aids in refolding denatured proteins, preventing damage (Vazzana et al., 2008; Tort et al., 2003). Both proteins contribute to immune activation by enhancing the function of immune cells such as macrophages and lymphocytes (Aneja et al., 2006). The study provides valuable insights into the immune responses of Asian seabass to *V. alginolyticus*, emphasizing the importance of understanding the temporal and tissue-specific dynamics of immune responses.

Chapter 4. Different vaccination modalities in Asian seabass against formalin inactivated *Vibrio alginolyticus*: pilot study

4.1. Introduction

Three primary vaccination methods are commonly used in aquaculture: immersion (bath and dip), injection (intraperitoneal (IP) and intramuscular (IM)), and oral administration (Adams et al., 2008). Each of these methods has its advantages and limitations (Mondal & Thomas, 2022). Understanding the comparative efficacy of these vaccination methods is crucial for optimizing disease control strategies in our study. This study aims to evaluate and compare protection efficacy and practical feasibility of immersion, injection, and oral vaccination methods against vibriosis in Asian seabass. By evaluating these parameters, the study aims to offer valuable insights that can inform the selection of the most effective vaccination strategy for controlling vibriosis in large-scale applications.

4.2. Materials and methods



4.3. Results

4.3.1. Protective Efficacy of Formalin-Killed Vaccine for Different Mode of vaccination

The survival percentages varied among the vaccinated groups based on the vaccination mode. The most favourable outcome was observed with intra-peritoneal vaccination, yielding a relative percent survival (RPS) of 60%. Similarly, intramuscular administration demonstrated

a commendable RPS of 53.3%. Oral vaccination exhibited a RPS of 20%, while bath and dip methods resulted in lower RPS rates of 16.6% and 10%, respectively.

4.4. Discussion

Intraperitoneal (IP) vaccination has consistently shown high efficacy in protecting fish against vibriosis, as demonstrated in multiple studies. The current study reported a 60% survival rate following IP vaccination with inactivated vaccines, supporting earlier findings. Kumaran et al. (2010) achieved a relative percent survival (RPS) of 75% in fish vaccinated with a formalin-killed *Vibrio anguillarum* vaccine, specifically targeting 20-30g fish. This suggests that IP vaccination is particularly effective for intermediate-sized fish. Similarly, Ahangarzadeh et al. (2023) reported an RPS of 86% in Asian seabass using a killed polyvalent vaccine, while Abu et al. (2020) observed an 87% survival rate in marine red hybrid tilapia vaccinated intraperitoneally with a formalin-killed *V. harveyi* vaccine. These findings indicate the strong immune responses induced by IP vaccination. Xu et al. (2019) further demonstrated that IP injection of an inactivated vaccine enhanced both humoral and innate immune responses in turbot, offering long-term protection.

Intramuscular vaccination, although effective, is slightly less potent than IP vaccination. The current study found an RPS of 53.3% for intramuscular administration. Kumar et al. (2007) developed a DNA vaccine for Asian seabass targeting the *V. anguillarum* OMP38 gene, achieving an RPS of 55.6%. Intramuscular vaccination has also been shown to produce significant immune responses, as evidenced by Liu et al. (2018), who reported an RPS of 90.67% in tiger puffer juveniles and 80.31% in adults using a live attenuated vaccine. Ji et al. (2008) observed a stronger immune response in *Seriola dumerili* vaccinated intramuscularly with *Vibrio hollisae* bacterin, where the RPS reached 75%. These results indicate that while intramuscular vaccination is effective, its performance may not consistently surpass IP vaccination, although it remains a viable option, particularly in specific contexts (Yang et al., 2009).

Immersion vaccination, while easier to administer, generally results in lower RPS. In the present study, immersion vaccination yielded RPS rates of 16.6% and 10%, reflecting suboptimal antigen uptake. However, innovative approaches have shown promise. Ototake et al. (1999) demonstrated that prolonged immersion improved protection, with no mortality in fish vaccinated against *Vibrio ordalii* compared to 58-77% mortality in brief immersion groups. Additionally, Galeotti et al. (2013) reported nearly 100% survival in European

seabass when combining immersion with an IP booster. These findings suggest that immersion vaccination can be improved, especially when used in conjunction with booster strategies.

Oral vaccination, despite its convenience for mass administration, typically produces lower RPS compared to injection methods. In the present study, oral vaccination achieved an RPS of only 20%. However, some studies have shown more promising results. Ram et al. (2019) reported an RPS of 85.4% in Asian seabass vaccinated orally with a biofilm vaccine against *V. anguillarum*, while Patil et al. (2014) demonstrated survival rates of 90.33% in *Fenneropenaeus merguiensis* when orally vaccinated with formalin-killed bacterins. Furthermore, Mohamad et al. (2022) achieved RPS rates of 70-80% in Asian seabass and hybrid grouper using an oral *V. harveyi* vaccine with double booster immunizations. These studies suggest that while oral vaccination may be less effective than injection methods, combining it with booster strategies could significantly improve outcomes.

Overall, intraperitoneal vaccination remains the most effective method for the present study, for our large-scale studies involving formalin-inactivated and recombinant vaccines, IP vaccination was selected. However, advancements in immersion and oral vaccination techniques, especially when combined with booster strategies, offer promising alternatives that warrant further exploration in future research.

Chapter 5. Development and assessment of inactivated *Vibrio alginolyticus* vaccines in Asian seabass

5.1. Introduction

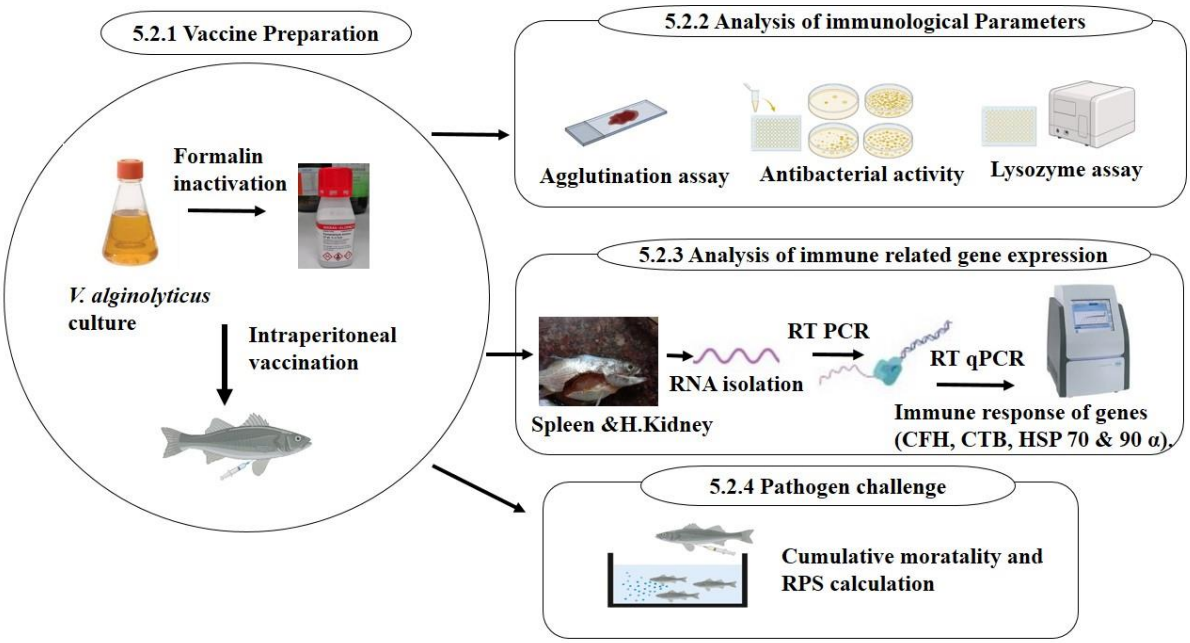
The development of effective vaccines against *V. alginolyticus* infections is crucial to mitigate economic losses and address public health concerns. Different types of vaccine technologies, such as live attenuated, subunit, and inactivated vaccines, are available (Ma et al., 2019). Among these options, inactivated vaccines have demonstrated several advantages for combating vibriosis in fish (Amir et al., 2022). Inactivated vaccines are produced by rendering pathogenic bacteria non-infectious using chemicals, heat, or radiation while maintaining their immunogenic properties. One of the major advantages of inactivated vaccines is their safety (Du et al., 2024). Since the bacteria in the vaccine are no longer infectious, there is no risk of causing disease in vaccinated fish or spreading to non-vaccinated fish. Additionally, inactivated vaccines offer stability, enabling long-term storage without loss of potency. This characteristic simplifies transport and administration, particularly in remote locations (Ma et al., 2019). The inactivated vaccines have paved way for a wider approach in development of commercial vaccines (Dadar et al., 2017).

Vaccines are essential for providing effective protection against infectious agents in aquaculture. To assess their efficacy, key biomarkers such as serum agglutination, bactericidal activity, and lysozyme levels are measured. Serum agglutination reflects the humoral immune response by indicating the presence of specific antibodies (Dhasarathan et al., 2014), while bactericidal assays evaluate both adaptive and innate immunity through the ability of antibodies and complement proteins to neutralize pathogens (Mokhtar et al., 2023). Lysozyme activity, which breaks down bacterial cell walls, highlights the activation of innate immunity (Biller et al., 2021). In tandem with these assays, the expression of immune-related genes, monitored through qRT-PCR offers valuable insights into the activation of protective mechanisms post-vaccination (Nguyen et al., 2017). This expression is critical for evaluating vaccine performance, particularly in triggering adaptive immunity and regulating inflammation via cytokine responses (Rebl & Goldammer, 2018). Together, these indicators provide a comprehensive understanding of vaccine-induced immune responses, ensuring protection against pathogens like *Vibrio* species.

The aim of the research was to evaluate and compare the effectiveness of adjuvanted and non-adjuvanted inactivated *V. alginolyticus* vaccines in protecting Asian seabass against vibriosis. The immune response in vaccinated fish was assessed by analyzing the expression of immune-responsive genes and measuring serum parameters. Additionally, the protective

efficacy of the various vaccine formulations such as adjuvanted and unadjuvanted were evaluated against *V. alginolyticus* infection through an intraperitoneal challenge.

5.2. Materials and methods



5.3. Results

5.3.1. Protective Efficacy of Formalin-Killed Vaccine

The vaccinated groups (T1 (without adjuvant) and T2 (with algel adjuvant) showed survival rates of 80% and 90%, respectively, compared to 26.6% in the non-vaccinated control group. The relative percent survival (RPS) was 72.7% for T1 and 86.5% for T2. Pathogen (*V. alginolyticus*) was isolated from all dead fish, confirming infection.

5.3.2. Agglutination Reaction

T1 and T2 groups exhibited significantly higher agglutination titers post-immunization compared to the control. Peak titers were observed at 2 weeks post-immunization (5 log2 in T2 and 4 log2 in T1), indicating a strong antibody response, particularly in the adjuvanted T2 group.

5.3.3. Antibacterial Activity of Sera

Sera from vaccinated groups (T1 and T2) demonstrated significant antibacterial activity against *V. alginolyticus*, reducing bacterial survival to ~77%, compared to 92.1% in the control group. The antibacterial efficacy peaked at 3 weeks post-vaccination.

5.3.4. Serum Lysozyme Activity

Lysozyme activity significantly increased in both vaccinated groups, peaking in the second week post-vaccination. The T2 group exhibited higher lysozyme activity than T1, highlighting the vaccine's effectiveness in stimulating innate immune responses.

5.3.5. Gene Expression Profiling

Complement Factor H (CFH): T1 showed upregulation in the spleen and head kidney tissues at 21 days post-vaccination (DPV). T2 had mixed results, with upregulation at some points but overall downregulation at 21 DPV.

Cystatin B (CSTB): T2 group showed significant upregulation in the head kidney at 7 DPV, while T1 exhibited a gradual increase over time.

HSP70: Both groups exhibited consistent upregulation in the head kidney at all time points, with T1 showing the highest expression at 21 DPV.

HSP90α: T2 group showed upregulation at 14 and 21 DPV in the spleen, while T1 exhibited fluctuating expression in both spleen and head kidney tissues.

These findings demonstrate that the formalin-killed vaccine, particularly in the adjuvanted T2 group, effectively enhanced immune responses, providing significant protection against *V. alginolyticus* infection in Asian seabass.

5.4. Discussion

Our study demonstrated that the intraperitoneal administration of the inactivated *V. alginolyticus* vaccine in Asian seabass, especially when combined with an adjuvant, provided substantial protection, achieving an 86.5% relative percent survival (RPS) after three weeks. This finding aligns with previous studies, highlighting the effectiveness of intraperitoneal injections for inducing immune responses and reducing mortality (Bromage & Owens, 2002; Ohtani et al., 2019). Intraperitoneal administration is preferred for its controlled delivery of vaccines and pathogens, as seen in other fish species, including grouper and tilapia (Evans et al., 2004; Huang et al., 2014). The adjuvant-enhanced group (T2) consistently showed superior immune responses across different immune parameters. Algel, the adjuvant used, is known for its ability to slowly release the antigen, thus prolonging immune stimulation (Lindblad, 2004; Sun et al., 2009). This improved response is consistent with previous findings that adjuvants enhance vaccine efficacy by boosting the immune system (Pellegrino et al., 2015).

Antibody titers, which peaked at three weeks post-vaccination, were significantly higher in vaccinated groups, especially in the T2 group. This aligns with research on other fish

vaccines, where adjuvants improved humoral responses (Nguyen et al., 2017). While antibody levels declined after the peak, they remained higher than in controls, indicating lasting immune memory, as observed in other studies (Cao et al., 2018).

Serum antibacterial activity against *V. alginolyticus* was significantly higher in vaccinated fish, especially the adjuvanted T2 group. The reduction in bacterial survival suggests enhanced immune activation, consistent with studies where vaccinated fish showed improved bacterial clearance (Nguyen et al., 2017). These results affirm the vaccine's ability to stimulate a potent humoral response, especially when adjuvants are used.

Increased serum lysozyme activity post-vaccination further supported the vaccine's effectiveness. The peak lysozyme activity at 14 days post-vaccination, especially in the T2 group, underscores its role in non-specific immunity. Lysozyme, a key antibacterial enzyme, has been shown to increase significantly in vaccinated fish, contributing to pathogen defence (Dong et al., 2013; Erfanmanesh et al., 2024). The ability of lysozyme to target both Gram-positive and Gram-negative bacteria emphasizes its importance in immune defence (Ferraboschi et al., 2021; Khorshidian et al., 2022).

Gene expression analysis revealed significant modulation of immune-related genes, such as complement factor H (CFH), Cystatin B, and heat shock proteins (HSP70 and HSP90α). CFH plays a critical role in regulating the complement system and maintaining immune homeostasis, as seen in other teleost fish (Encinas et al., 2010; Nakao et al., 2011). Upregulation of CFH in vaccinated fish suggests its involvement in the immune response against *V. alginolyticus* infection. Similarly, the transcriptional modulation of Cystatin B, particularly its upregulation, indicates its role in antibacterial defense, as reported in other teleosts (Magister & Kos, 2013). The upregulation of HSP70 and HSP90α in vaccinated fish, particularly in the spleen, highlights their role in stress responses and immune regulation. These findings are consistent with previous studies showing HSP's involvement in immune responses to infections and stressors (Mohd et al., 2013; Yang et al., 2020). However, the tissue-specific responses of these proteins indicate the complexity of their regulation, warranting further investigation.

In summary, the inactivated *V. alginolyticus* vaccine, particularly when combined with an adjuvant, significantly enhances immune responses in Asian seabass, providing strong protection against infection. These findings offer valuable insights for future vaccine development strategies in aquaculture.

Chapter 6. Development and assessment of recombinant *Vibrio alginolyticus* GAPDH vaccines in Asian seabass

6.1. Introduction

Recombinant vaccines, in particular, have gained significant attention due to their ability to provide targeted and effective protection (Du et al., 2022). Compared to traditional formalin-inactivated vaccines, recombinant vaccines present several advantages. They allow the expression of specific antigens, resulting in a more focused immune response and reducing the risk of introducing potentially harmful components from the inactivated pathogen. This significantly enhances safety, especially when dealing with pathogens that are difficult to cultivate or pose carcinogenic risks (Ji et al., 2020). Additionally, recombinant vaccines can be produced in a more controlled environment, leading to improved vaccine consistency (Tammas et al., 2024). However, they also have certain limitations, such as the requirement for adjuvants to enhance immune responses and the higher costs associated with their development (Ji et al., 2020). Studies have shown that adjuvanted recombinant aquatic vaccines demonstrate superior efficacy compared to non-adjuvanted counterparts (Moni et al., 2023).

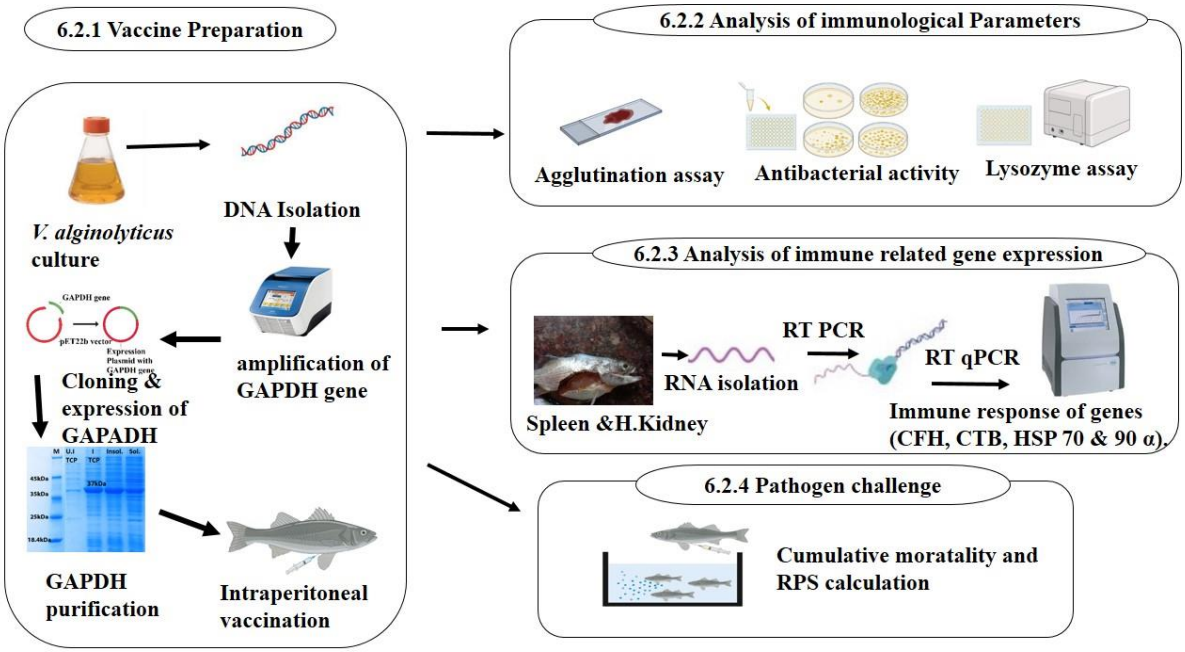
A promising candidate antigen for recombinant vaccination against vibriosis is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a crucial metabolic enzyme involved in glycolysis (Perez & Potter, 2016). Beyond its metabolic role, GAPDH has been found to possess additional functions, including binding to extracellular matrices and modulating host immune responses. These properties suggest its potential involvement in virulence and surface localization, making it an attractive target for vaccine development against various pathogens. Research has revealed the presence of immune responses against GAPDH in numerous microorganisms, further supporting its potential as a vaccine candidate, particularly for pathogens like *Schistosoma mansoni* (Goudot et al., 1989) and *Echinococcus multilocularis* (Müller et al., 2001). GAPDH's high degree of conservation across different species is another compelling reason for its consideration. This conservation suggests that a vaccine based on GAPDH from one microorganism could potentially offer cross-protection against unrelated pathogens, as observed with certain fish pathogens (Li et al., 2012, An et al., 2023).

To assess the efficacy of recombinant vaccines, various serum parameters, including serum agglutination assays, serum antibacterial activity, and serum lysozyme assays, are commonly evaluated. These parameters offer insights into the humoral immune response, indicating the vaccine's ability to stimulate antibody production and enhance antibacterial defences (Zhang

et al., 2023). Furthermore, the expression of immune-related genes in vaccinated fish, such as those involved in cytokine production and immune regulation, serves as another vital indicator of vaccine performance. Monitoring these gene expression profiles helps elucidate how recombinant vaccines modulate both innate and adaptive immune responses (Silvaraj et al., 2020).

The primary objective of this study is to evaluate the efficacy of a recombinant GAPDH vaccine against *V. alginolyticus* in Asian seabass. This will involve comparing its performance to formalin-killed vaccines, assessing the impact of different adjuvants, analyzing serum parameters, and monitoring immune-related gene expression.

6.2. Materials and methods



6.3. Results

6.3.1. Recombinant Expression of *V. alginolyticus* GAPDH

The *V. alginolyticus* GAPDH gene was successfully cloned and expressed in *E. coli* BL21 using the pET22b vector. The recombinant protein (37 kDa) was induced with Isopropyl-β-D-thiogalactopyranoside (IPTG) and purified with high purity using NGC chromatography, confirmed by SDS-PAGE analysis.

6.3.2. Protective efficacy of the vaccines

Immunized fish groups showed varying protection levels. The highest survival rate (100%) was observed in the group vaccinated with high protein concentration and adjuvant (V4).

Lower survival rates were seen in groups with low protein or no adjuvant, with V1 (low protein, no adjuvant) showing 57% survival, in contrast, V2 (low protein with adjuvant) and V3 (high protein without adjuvant) shows same survival rate (71.5%).

6.3.3. Agglutination Reaction

Serum agglutination titers were significantly higher in all vaccinated groups compared to the control. The V4 group (high protein with adjuvant) showed the highest agglutination titer, peaking at 5 log2 by the second week and remaining stable. Other groups exhibited lower titers with declines in the third week.

6.3.4. Antibacterial Activity

Sera from vaccinated fish reduced bacterial counts of *V. alginolyticus* on TCBS plates. The V4 group showed the greatest bacterial reduction by the third week (79.8%), followed by V3 and V2 groups (81.8% and 84%). The V1 group peaked at the second week (84%) but showed reduced defense by the third week.

6.3.5. Serum Lysozyme Activity

Lysozyme activity increased progressively in all vaccinated groups, peaking at 21 DPV. The highest lysozyme activity was seen in the V4 group, with V1 and V2 showing significantly lower activity. The control group showed no lysozyme activity throughout the study.

6.3.6. Gene Expression Profiling

Complement Factor H (CFH): Highest expression was observed in the spleen of the V4 group at 21 DPV. V1 and V2 showed early upregulation but downregulated by 21 DPV. In the head kidney, CFH was maximally upregulated in V3 at 21 DPV, while V4 showed gradual upregulation. Cystatin B: Peak expression in the V3 group at 7 DPV in the head kidney, with V4 showing consistent expression in both spleen and kidney. HSP70 and HSP90: Both genes showed higher expression at 21 DPV in V3 and V4, especially in the spleen and head kidney, indicating a strong stress response in these groups.

Overall, high protein concentration with adjuvant (V4) consistently outperformed other groups, showing superior immune responses across multiple parameters.

6.4. Discussion

Vibriosis, caused by pathogens such as *V. alginolyticus* and *V. parahaemolyticus*, is a major disease impacting marine aquaculture, especially in species like Asian seabass (Ina-Salwany et al., 2019). Vaccination has emerged as a crucial strategy to reduce antibiotic dependency in controlling vibriosis (Ma et al., 2019). While formalin-killed bacterins have been the traditional method, their variable efficacy across species has prompted research into alternative vaccine types (Ji et al., 2020). Recent developments include live attenuated, DNA,

and subunit vaccines, which offer enhanced protection (Liu et al., 2011; Hu et al., 2012; Pang et al., 2018; Zhou et al., 2020). Among these, recombinant subunit vaccines have gained attention for their safety and cost-efficiency, particularly those targeting immunogenic proteins like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ji et al., 2020). In this study, four recombinant GAPDH (rGAPDH) vaccine formulations were evaluated in Asian seabass, with the adjuvanted high-dose vaccine (V4) yielding 100% survival. This superior protection, in line with studies using adjuvants like aluminum hydroxide gel, demonstrates the enhanced immunoprotective effect of adjuvanted recombinant vaccines (Jiang et al., 2015; Galindo et al., 2019; Dar et al., 2021). In comparison, non-adjuvanted formulations offered lower relative percent survival (RPS), similar to findings with *E. tarda* in Japanese flounder (Liu et al., 2005). This highlights the potential of adjuvants to boost vaccine efficacy, particularly aluminum-based ones, which show strong, though variable, performance across different species and pathogens (He et al., 2014; Sun et al., 2022).

Serum biomarker assays, such as agglutination, bactericidal activity, and lysozyme activity, provided key indicators of the immune response in vaccinated fish. The V4 group exhibited peak agglutination titers of 5 log2, reflecting a robust humoral response (Silvaraj et al., 2020). This response was accompanied by high bactericidal activity, with V4 sera reducing bacterial counts by 79.8%, paralleling results from similar studies (Pham et al., 2021). The V4 group also showed elevated lysozyme activity by the third week, aligning with trends observed in recombinant HSP-based vaccines (Pham et al., 2021; Dar et al., 2021). However, long-term studies suggest the need for extended monitoring to fully understand the persistence of these immune responses (Dar et al., 2021).

Gene expression analysis revealed key insights into immune regulation post-vaccination. Complement factor H (CFH) expression patterns in the V3 and V4 groups indicated a sustained immune response, potentially linked to higher survival rates (Qi et al., 2018; Wei et al., 2022). Similarly, the expression of Cystatin B (CTB) and heat shock proteins (HSP70 and HSP90 alpha) highlighted the importance of these genes in modulating both innate and adaptive immunity. High expression of CTB in the V3 and V4 groups, coupled with increased HSP expression at 21 days post-vaccination (DPV), suggested a stronger and more durable immune response, echoing findings in other fish species (Kodagoda et al., 2022; Ali et al., 2024; Josepriya et al., 2015).

In conclusion, recombinant GAPDH vaccines, especially when adjuvanted, offer a promising solution for controlling vibriosis in Asian seabass. Further research into antigen selection and long-term immune responses will be crucial for optimizing vaccine efficacy in aquaculture.

Chapter 7. Summary and conclusion

Aquaculture is essential in meeting the global food demand, particularly in providing high-quality protein and nutrients to growing populations, especially in economically challenged regions. Asian seabass (*Lates calcarifer*), one of the most commercially important fish species, is widely cultured due to its increasing demand, attributed to its tender and flavorful meat. However, the aquaculture industry is severely threatened by vibriosis, a bacterial disease caused by various *Vibrio* species, with *V. alginolyticus* being a predominant pathogen. Since its identification as a major pathogen in India from 2001, *V. alginolyticus* infections have posed significant challenges, causing high mortality rates in Asian seabass. The use of antibiotics is the primary treatment option for controlling vibriosis, but it poses significant risks, including the development of antibiotic resistance and environmental harm. Although some commercially available inactivated vaccines exist, they have limitations in terms of efficacy and long-term protection. It is crucial to prioritize the development and availability of effective vaccines for farmers to raise disease-free and healthy fish, ensuring a sustainable food supply. By advancing vaccine technology, we can ultimately eliminate the reliance on antibiotics in aquaculture, paving the way for healthier future generations and environmentally responsible fish farming practices.

This research focuses on the development and comprehensive evaluation, both at the molecular and immunological levels, of inactivated and recombinant vaccines to control *V. alginolyticus*. The goal is to reduce antibiotic dependence and enhance fish health by providing effective immune protection, thereby promoting sustainable aquaculture practices. Through detailed analysis of immune responses and gene expression, this study aims to contribute to more robust and long-term solutions for managing vibriosis in Asian seabass.

Key Findings of the Study:

1. **Whole-genome sequencing of *Vibrio alginolyticus* (CMFRI-JF260912)**:
    - Two circular DNA chromosomes were identified through whole-genome sequencing, providing insights into the virulence and antimicrobial resistance mechanisms of *V. alginolyticus*. Chromosome I contained 3,790 predicted ORFs, and Chromosome II had 2,065 predicted ORFs, with various virulence factors such as adhesion, biofilm formation, and toxin production.
    - Four antimicrobial resistance genes were detected, showing resistance to tetracycline, chloramphenicol, carbenicillin, and oxytetracycline. This highlights the need for effective vaccines to mitigate the impact of antibiotic-resistant *Vibrio* strains.

- Comparative analysis with other *Vibrio* species showed a close phylogenetic relationship between *V. alginolyticus* and *Vibrio natriegens*

2. **Critical infection period and histopathological changes**:
   - An experimental infection model was developed to determine the critical infection window of vibriosis caused by *V. alginolyticus*, revealing that significant histopathological changes occurred in the spleen, head kidney, liver, and gills within 12-48 hours post-infection.
   - The most severe damage was observed in the kidney, followed by the spleen and liver, with minor changes in the gills. Mortality rates reached 90–95% within this period, highlighting the aggressiveness of the pathogen.
   - The results emphasize the need for rapid intervention during this critical infection period to prevent severe organ damage and high mortality in infected fish.
3. **Transcriptomic profiling of immune-related genes**:
   - A comprehensive transcriptomic analysis of the spleen and head kidney was conducted at 12 and 24 hours post-infection, identifying 91,819 unigenes. The transcriptome sequencing yielded an N50 length of 1,515 bp, with the majority of differentially expressed genes (DEGs) being associated with immune responses.
   - Four immune-related genes-Complement Factor-H, Cystatin B, Heat Shock Protein 70 (HSP70), and Heat Shock Protein 90α (HSP90α)-were upregulated in response to infection, providing valuable molecular markers for immune responses in fish.
   - Real-time PCR validation confirmed the expression levels of these four genes, indicating their potential use as molecular markers for monitoring vibriosis/bacterial infection and vaccine efficacy in future studies.
4. **Development of inactivated and recombinant vaccines**:
   - Two primary vaccine types were developed: the formalin-inactivated whole cell vaccine and the recombinant subunit vaccine (rGAPDH). Various vaccination routes were tested, including oral, bath, dip, intramuscular, and intraperitoneal methods.
   - Intraperitoneal vaccination was identified as the most effective route, providing better immune protection and higher survival rates compared to

other methods. This route was selected for subsequent experiments due to its practicality and effectiveness.

- The study explored both inactivated and recombinant vaccine approaches, providing a comparative evaluation of the immune responses they triggered in Asian seabass.

5. **Formalin-inactivated vaccine efficacy**:
   - The formalin-inactivated vaccine was formulated with and without an adjuvant (algel). The algel-adjuvanted vaccine significantly improved the relative percent survival (RPS) to 86.5%, compared to 72.7% for the non-adjuvanted vaccine.
   - Algel was identified as an effective adjuvant, enhancing the immune response, including the upregulation of immune-related genes and an increase in antibacterial and lysozyme activities. This supports the use of algel in future vaccine formulations.
   - Gene expression profiling revealed that the formalin-inactivated vaccine effectively stimulated immune responses at multiple time points, with significant increases in antibody production and lysozyme activity, crucial for combating bacterial infections.
6. **Recombinant GAPDH subunit vaccine development**:
   - The recombinant GAPDH antigen protein was successfully produced and formulated into a subunit vaccine, with four dosages tested: rGAPDH 5 µg/200 µl (V1), rGAPDH 5 µg/200 µl with algel (V2), rGAPDH 10 µg/200 µl (V3), and rGAPDH 10 µg/200 µl with algel (V4).
   - The highest efficacy was observed in the group receiving 10 µg of rGAPDH with algel (V4), achieving 100% RPS. This group also showed the highest antibody levels and lysozyme activity, supporting the potential of this formulation as a candidate for future vaccine development.
   - Algel again proved to be a potent adjuvant, enhancing the immune response and providing superior protection compared to non-adjuvanted formulations.
   - Real-time PCR analysis of mRNA expression in the spleen and head kidney revealed the overexpression of Complement Factor-H, Cystatin B, HSP70, and HSP90α, particularly in groups V3 and V4 (recombinant vaccines) at 21 days post-vaccination.

- This enhanced gene expression correlated with stronger immune responses, suggesting that these genes play a key role in disease resistance and could serve as molecular markers for vaccine efficacy.
- The downregulation of these genes in non-vaccinated and less effective vaccine groups (V1 and V2) highlights the importance of optimizing vaccine formulations for enhanced protection.

Conclusion

The studies on *V. alginolyticus* provide a comprehensive understanding of its genetic makeup, infection dynamics, immune response, and vaccine development strategies. Whole-genome sequencing identified key virulence factors and antimicrobial resistance genes, emphasizing the challenge posed by antibiotic-resistant strains. Critical infection period analysis revealed rapid and severe organ damage, particularly in the kidney, highlighting the urgency for early interventions. Transcriptomic profiling pinpointed specific immune-related genes that could serve as biomarkers for infection and vaccine response. The development of inactivated and recombinant vaccines, with intraperitoneal vaccination being the most effective, offers promising tools for controlling vibriosis in Asian seabass. The efficacy of the currently developed vaccines (formalin inactivated and recombinant vaccines) studied by immune gene expression revealed that both the vaccine could elicit innate and adaptive immune response. Algel was identified as an effective adjuvant for both vaccine types, enhancing immune responses and providing superior protection. Real-time PCR validation of key immune-related genes offers a reliable molecular tool for future vibriosis studies and vaccine efficacy assessment and can be further used in gene expression studies and immune response studies to develop better health strategies in the disease management of Asian seabass aquaculture. This study successfully developed and evaluated both formalin-inactivated and recombinant subunit vaccines against *V. alginolyticus* in Asian seabass. The recombinant GAPDH vaccine, particularly in combination with algel adjuvant, demonstrated the highest survival rates (100% RPS), antibody production, and lysozyme activity, indicating its potential as a powerful tool for disease control in aquaculture, it can be develepoed as a commercial vaccine in future. However, additional field-level research is necessary to observe the marketability and viability of these vaccinations from an economic standpoint. This research provides critical insights into the immune mechanisms of Asian seabass and presents promising avenues for advancing vaccine development in aquaculture, contributing to sustainable fish farming and reducing the reliance on antibiotics for disease control.

Published/accepted/communicated articles:

1. **Azhar Shahansha T.S**., Pradeep M.A., Krupesha Sharma S.R. and Sanil N.K (2024). "Whole genome sequencing of *Vibrio alginolyticus*: Insights into virulence and antimicrobial resistance. Fishery Technology**.**
2. **Azhar Shahansha T.S**., Pradeep M. A, Krupesha Sharma S. R, Sreenath K R, Suja Gangadharan, Aswathy Joshy, Sanil N. K. (2024). Sequential development of histopathological manifestations in response to experimental *Vibrio alginolyticus* infection in Asian seabass. Journal of the Marine Biological Association of India.

Posters/papers presented:

1. **Azhar Shahansha T. S**, Pradeep M. A, P Ramesh Kumar, Sanil N. K (2023). Protective Efficiency and Immune Response of a Formalin inactivated Vaccine against *Vibrio alginolyticus* Infection in Asian Seabass (*Lates calcarifer (Bloch, 1790)*). 35th Kerala Science Congress held at MBC College, Idukki, Kerala, India, from February 10th to 14th 2023.

2. **Azhar Shahansha T. S**, Pradeep M. A, Sanil N. K (2023). Transcriptome analysis of immune response in Asian sea bass (*Lates calcarifer*) spleen tissue against *Vibrio alginolyticus* infection. International conference on recent advances in biological Sciences 2023, held at Kerala University, Trivandrum.

3. **Azhar Shahansha T. S**, Pradeep M. A, Krupesha Sharma S. R, Suja Gangadharan, Aswathy Joshy, Nidhiya Kottayi, Nivya A, Nisha E. A. and Sanil N. K (2020). Histopathological manifestation in Asian Seabass, *Lates calcarifer* (Boch, 1790) experimentally infected with *Vibrio alginolyticus.* International symposium "Marine Ecosystem Challenges and Opportunities" held at Kochi, Kerala, India from 7th to 10th January 2020 hosted by CMFRI Kochi.

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Research Guide

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